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Strategies for triggered drug release from liposomes: In search of "smart" lipids and "smart" triggers.

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Long circulating liposomal drug carriers are widely used in experimental cancer therapy because they avoid excretion and benefit from the EPR-effect to accumulate at the tumor site while simultaneously limiting systemic exposure to the cytotoxic drug. New insights in lipid behavior have led to such stable carriers that despite increased accumulation, the unloading of the drug at the target site is very poor. This opens up a new challenge to trigger drug release at the target site, while still retaining most of the drug inside the carrier while it resides in the bloodstream. Liposomes are very suitable to design these kinds of triggered release systems, because of the diversity of lipids and versatility of lipid membranes. Triggers can be intrinsic, i.e. located at the tumor site, or applied from the outside. Intrinsic triggers such as the acidic environment at the tumor, or the increased expression of phospholipases, and applied triggers such as radiation, light or heat can destabilize the membranes of smartly designed liposomes. Creating a triggered release system will always be a trade-off between systemic stability and susceptibility to the trigger. However, sophisticated triggering systems, that combine a trigger with an advanced imaging system, can further optimize the efficacy of targeted triggered release.

Nanoparticulate drug delivery systems have become an integral part of the research in anti-cancer treatment. There are multiple reasons why nanocarriers are attractive for drug delivery. For instance, they can help to increase the solubility of low-soluble drugs, or protect vulnerable compounds from degradation in the bloodstream. But more importantly, traditional anti-cancer drugs have significant side-effects that can be lowered by encapsulating them in a drug carrier. The strategy to reduce systemic distribution and selectively target the nanocarrier to the tumor site aims to maximize drug concentration inside the tumor and to limit exposure of surrounding tissue to the cytotoxic drug. This allows for the use of much higher doses while at the same time limiting systemic side-effects[1–4]. The drug delivery systems can be coupled to active targeting ligands to direct them to cell surface receptors that are unique to, or overexpressed by the cancer cells[5,6]. This is the concept of the 'Magic Bullet', as proposed by Paul Ehrlich already in 1906; A 'bullet' loaded with a suitable drug to treat the disease that is 'magically' sent to the target site (tumor site)[5]. A wide variety of targeting ligands has been identified and tested, mainly targeted to growth-factor related receptors, amongst which the folate receptor[7–9], anti-Her2 targeted particles in breast cancer[10–12], VEGFR-targeted anti-angiogenic systems[13–15] and many more.

However, the effect of active targeting is negligible when compared to passive targeting. In fact, there would be no active targeting without passive targeting[16,17]. Before the targeting ligand can bind the cell surface receptor, it first has to reach the target site and this is mainly driven by the EPR (Enhanced Permeability and Retention) effect[18–20]. Because tumor tissue divides rapidly and uncontrolled, it has an poorly developed vasculature system that lacks secondary structure and has wide fenestrations between the endothelium cells which makes the tumor blood vessels leaky[21]. Nanoparticles can extravasate through these gaps in the endothelium and accumulate in the tumor interstitium. After this, the targeting ligands can recognize and bind their target receptors, but this does not add anything to the amount of carriers that is actually delivered to the tumor site. It was shown that the targeted form of a nanocarrier reached the tumor site in the same amount as the non-targeted counterpart. Furthermore, the distribution profile was the same, regardless of whether the target receptor was expressed by the tumor or not[17,22,23]. This means that there is no real need for active targeting of drugs that can cross the target cell membrane on their own. However, nanocarriers also have the ability to help biomacromolecules like peptides, proteins and nucleotides cross the cell membrane, while they are unable to do so on their own due to unfavorable characteristics such as size and charge[23,24].Targeting ligands interact with surface receptors and molecules that initiate all kinds of internalization mechanisms for intracellular drug delivery[24–26]. So, when internalization is required, active targeting ligands help the delivery, but as far as localization to the tumor area is concerned, this is almost exclusively caused by the EPR effect.

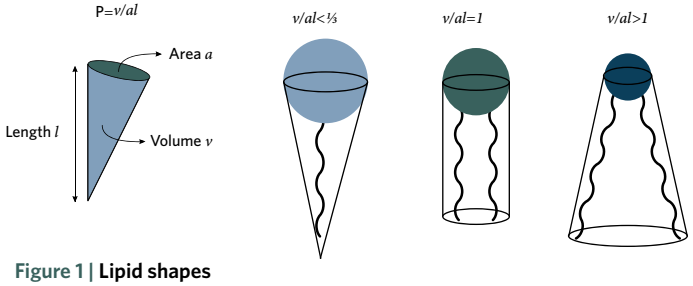

Figure 1 | Lipid shapes
Lipids can be classified based on their shapes, that are mainly determined by the relative size of the headgroup and acylchain(s).

The EPR effect is highly dependent on molecular weight because in general, the bigger the MW, the lower the clearance and the longer the circulation time, which is necessary for the particle to permeate to the tumor[27]. All particles with a weight above 40 kDa are thought to benefit from the EPR effect as this is the threshold for renal clearance and this effect has been observed for particles ranging from 10-500 nm[19,21,28,29]. But renal clearance is not the only way particles can be removed from the bloodstream; they must also avoid the reticuloendothelial system (RES). Macrophages of the RES residing in the liver and the spleen are also a big contributor to the clearance of the particles from the circulation[30–35]. This obviously results in a low availability of the carrier in the bloodstream, but may also cause toxicity to the RES and immune system because of the accumulation of the cytotoxic drug in the liver and the spleen if it is not broken down in the lysosomes[30,31]. A commonly used strategy to avoid the RES is to graft the surface of the particles with a polymer that shields it from interaction with macrophages and surface receptors. PEGylation, with poly-ethylene glycol is the most widely used method to create long circulating "stealth" nanocarriers[10,32–39], but other hydrophilic polymers are equally suitable, like poloxamers[40,41], dextran[14,42], chitosan[12,43,44] or most recently, squalene[45,46]. These surface modifications protect them from clearance by the kidneys because of the added MW and from the RES by masking the reactive surfaces[32,33]. It also improves the stability and biodistribution due to the more hydrophilic nature. The longer circulation time and better stability allow them to extravasate to the tumor site and stay there for up to a couple of days[34,35]. And this is exactly the problem with many of the nanocarrier systems: they reach the target location intact and have been able to retain their payload in the circulation and subsequently stay in the target tissue but are so stable that they do not release their contents. This is probably best illustrated by the fact that the landmark formulation Doxil® (=Caelyx® outside the USA), a PEGylated liposomal formulation of doxorubicin, got FDA-approval based on a superior safety-profile, rather than improved efficacy, as compared to free doxorubicin[47,48]. The importance of reducing side-effects should not be underestimated, but this does indicate that despite the higher drug load, intratumoral drug levels are not increased. It is a well-known problem with many of the commonly used carrier systems is that they either have a high burst-release in the circulation (e.g. in PLGA nanoparticles)[49,50] or an incomplete release at the target site[51–53].This calls for a new approach that releases the drug load at the tumor site, while retaining the encapsulated drug in the circulation.

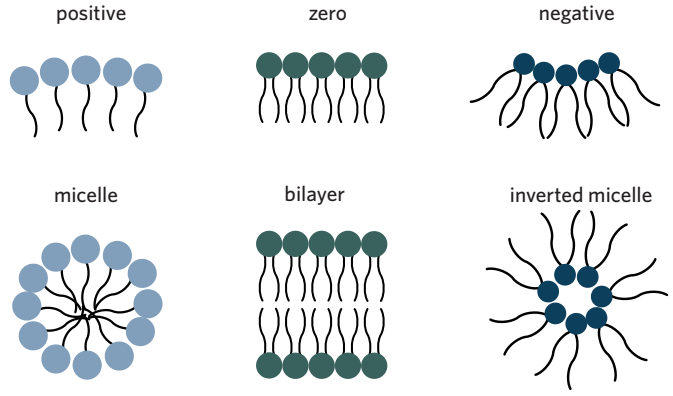

Figure 2 | Membrane curvature and preferred conformation
Lipids that have a conical shape increase the membrane curvature and prefer a (inverted) micellar conformation. Cylindrical shaped lipids have zero curvature stress and form stable bilayers.

In order to create delivery systems that release their contents to the tumor area, there are three prerequisites. First, it must respond to a trigger, to quickly and completely release its content when it has reached the tumor. This trigger can be an intrinsic trigger in the tumor microenvironment, or an extrinsic trigger that is applied to the tumor site. Secondly, the release in the circulation should be minimal. The carrier system must be long circulating and stable and avoid release of the drug content before it reaches its location. And finally, because the release is outside the cell, the drug substance must be able to penetrate the cell membrane on its own (or work outside the cell).

Liposomes are very suitable to create triggered release systems. New insights in lipid behavior and physico-chemical properties have enabled stable carriers that release their contents upon a tumor-specific trigger.

Lipid polymorphisms and phase behaviour

Liposomes were first discovered by the hematologist Dr. Alec Bangham in the early 1960's in an attempt to visualize lipid structures under the electron microscope[54,55]. Liposomes are defined as vesicles consisting of lipid bilayers that self-assemble in water as a result of hydrophobic interactions. These can be single lamellar bilayers or multiple concentric bilayers. They can be used to incorporate water-soluble drugs in the aqueous core of the liposome. There are many bilayer forming lipids, but there are also numerous other lipid structures[56]. Understanding lipid polymorphisms is essential to create a stable carrier and to exploit their properties to create a triggered release system.

Lipids are amphiphilic molecules that consist of a hydrophilic headgroup and one or two hydrophobic tailgroups that are the main driving force of their phase behavior. They can be classified based on their shape, namely conical shape (e.g. fatty acids FA, lysolipids LL), cylindrical shape (e.g. phosphatidylcholines PC) and inverted conical shape (e.g. phosphatidylethanolamines PE) *(See Figure 1)*. The reason that these lipids assemble in closed structures or phases, is to minimize exposure of the hydrophobic tails to water, which makes them cluster together. Ideally, this results in bilayer formation, as we see in all biological membranes. However, when lipids are more conically shaped, membrane curvature is increased. When these lipids are forced into bilayers, they suffer from a certain amount of curvature stress because the hydrophobic tails are more easily exposed, which is energetically unfavorable. When the curvature stress gets too high, the system can "flip" to another phase, and form e.g. micelles (positive membrane curvature) or inverted micelles (negative membrane curvature)[56,57]. *See Figure 2.*

The phase behavior can be predicted by comparing the surface of the polar group relative to the apolar tail. This is defined more exactly in the Israelachvili–Mitchell–Ninham packing parameter: $P=v/al$ Where v is the molecular volume, a is the diameter of the polar headgroup and l is the length of the molecule[58] *(See Figure 1)*. Cylindrical shaped lipids have a P value of close to 1 and are perfect for bilayer forming. When P>1, the hydrophobic body is much larger than the polar head, resulting in inverted micelle formation. When P<1/3, the polar headgroup has a much bigger volume than the body, what makes these lipids adopt a normal micelle form, with the polar heads facing outward[58,59]. Of course there are also intermediate forms, such as the cubic phase, hexagonal phase or inverted hexagonal phase, that form as a result of the lipids compartmenting the water in hydrophilic structures while limiting exposure of the hydrophobic tails[56,58–60].

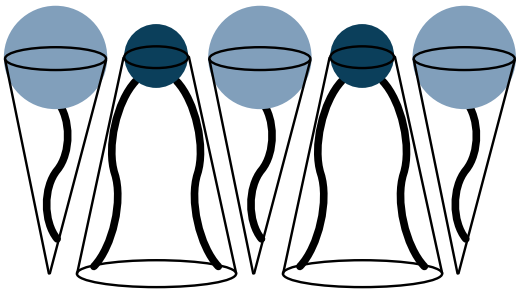

Figure 3 | Complementing lipid shapes
When lipids with complementing lipid shapes are combined, the membrane curvature stress is relieved and a bilayer can be formed.

Mixing different types of lipids with complementing shapes also results in intermediate forms. When a lipid with a negative membrane curvature is mixed with a lipid that has a positive membrane curve, depending on the ratio, the membrane curvature can approach zero and form a stable bilayer. *See Figure 3.*

The most widely used non-bilayer forming lipid is dioleoylphosphatidylethanolamine (DOPE), that, due to its unsaturated chains, has an inverted conical shape and therefore adopts the inverse hexagonal phase in isolation[61–66]. It can be forced into a bilayer by conical shaped membrane stabilizing lipids, like PEG-linked lipids[66], cholesterol-hemisuccinate (CHEMS)[64], oleic acid[65] or phosphatidylserines (PS)[63]. All stabilizing lipids have a cone shape because of the relative bulkiness of the head group that in the case of CHEMS, oleic acid and PS is a protonatable group. This means that at lower pH (when the group is protonated and uncharged) the cone shape and thus the membrane stabilizing ability is lost and contents are released . This has been exploited to create pH-sensitive "fusogenic" liposomes to allow endosomal escape[61,67]. This is of importance for intracellular delivery of oligonucleotides and is excellently reviewed by Fattal *et al*[61]. This mechanism also has been used to deliver cargo to the acidic environment of the tumor and will be addressed in the next chapter.

Relieving membrane curvature stress is important to be able to form bilayers, but these membrane stabilizing lipids also help to order the lipid membrane. A lipid bilayer is not static but is a dynamic surface. Lipids can diffuse over the lipid plane and the faster they do this, the more fluid the membrane[68,69]. Lateral diffusion correlates with the ordering of the lipid tails. When the tails are tightly packed, they are neatly ordered and only little diffusion is possible, making the membrane more rigid. This is called the solid-ordered state or "gel" phase. When there is a lot of diffusion of the lipids, the tails are less organized and the membrane is more fluid and has a higher permeability. This is called the liquid-disordered state or "fluid" phase[69]. Cholesterol is a well-known membrane stabilizer that was found to induce a change to the intermediate liquid-ordered phase and hence a better organization in the lipids, making the membrane less fluid and more rigid[51,68,70–72]. *See Figure 4.* This is even thought to be an important step in evolution. Alec Bangham himself already proposed that primitive cellular life forms could be housed in liposome-like structures[55]. When chemical conditions changed so that cholesterol could be synthesized, it allowed eukaryotic membranes to better retain shape and function and to protect their important proteins and organelles from the outside environment[56,70].

Another factor in membrane fluidity and elasticity is the phase transition temperature (Tm)[73]. Lipids diffuse faster at higher temperatures until they "melt" and change phase (from solid to liquid phase) at a certain temperature[59,68,69,74,75]. This temperature is mainly dependent on the length and saturation of the lipid tail[74,75]. Generally, lipids with a high Tm are more tightly packed because the phase equilibrium shifts to the gel phase. In order to have a solid-ordered phase (rigid and impermeable) membrane at physiological temperature, the Tm should be above 37°C[51]. Apart from the length, number and saturation of the acyl chain, the size and ion strength of the headgroup also influence the Tm[73]. These parameters can be tailored to make a more stable carrier or used to trigger its release, as is described in the section about hyperthermia-mediated drug release. Early research has mainly focused on finding mixtures of lipids that enhance stability resulting in such stable formulations that the unloading is now often very poor.

The challenge is to design the lipids in such a way that they are still stable in the circulation, but destabilize the bilayer at a certain trigger. This can be a trigger at the tumor site, or an outside trigger that is applied when the liposomes have reached the tumor site, or that is applied only to the tumor site. The following chapters describe examples of how lipids are engineered to respond to an intrinsic or extrinsic trigger.

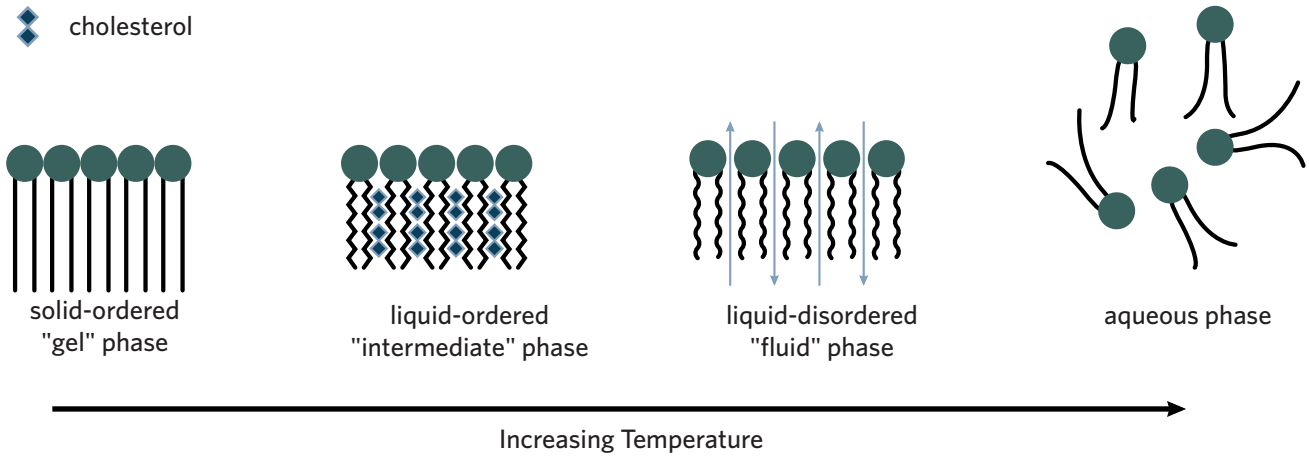

Figure 4 | Phase transitions with increasing temperatures
At temperatures below the phase transition temperature (Tm), lipid chains are fully stretched and tightly packed, resulting in a very stable membrane in the solid-ordered phase. At higher temperatures, the chains start to melt and the membrane becomes fluid and leaky in the liquid-disordered phase, or the membrane completely dissolves at high temperatures where the chains are fully melted. An intermediate phase, where the lipid chains are more fluid but still ordered (liquid-ordered), is created when cholesterol is incorporated in the membrane.

Acidosis induced release from pH sensitive liposomes

One of the first attempts to actively release the drug load in tumor specific conditions made use of the slightly acidic environment around a tumor. The high glycolysis rate of the fast proliferating cells in combination with the slightly hypoxic areas in poorly perfused tumor areas generates lactic acid (pK_a 3.9) under anaerobic conditions[76–78]. This lowers the extracellular pH of the tumor microenvironment with more than 0.5 pH units and tumor cells are remarkably resistant to these conditions. They even show a maximum proliferation rate at pH<7, a condition under which normal cells usually undergo apoptosis[76–80]. Consequently, the edge of the tumor acts as an acidic wave, killing healthy tissue and making room for tumor proliferation[80,81]. In this way, the high dependence on anaerobic metabolism can even be seen as a Darwinian adaptation, rather than a necessity due to hypoxia[76,77]. In vivo pH measurements showed a correlation between lowering pO_2 and pH (indicating a shift to anaerobic glycolysis) which in turn negatively correlated with the distance from the supplying blood vessel76,82,83. Surprisingly, also well-perfused areas showed a preference for anaerobic glycolysis, which again indicates that this is an evolutionary "choice" rather than a necessity[76,83]. This preference for anaerobic glycolysis is known as the 'Warburg Effect'[78]. The average extracellular pH in a tumor of 6.83 (range, 6.72-7.01; n=268)[84,85] is quite significant for metabolism and proliferation as mentioned above, but it is a rather small window to exploit for an active release trigger. The liposomes should be fully stable at physiological pH (7.4) and destabilize at 0.6 pH units lower. Weak acidic or basic lipids need a pK_a-pH gap of 4 units to fully associate or dissociate making the tumor pH of limited applicability.

The previous chapter already mentioned the use of pH-sensitive membrane stabilizing lipids. This approach uses a protonatable lipid that loses its stabilizing abilities when it is protonated, which makes the hydrophilic head smaller (Conical shape changes to more cylindrical; P value increases). The first publication in Science (1980) by Yatvin *et al.* used palmitoylhomocysteine (PHC) as stabilizing lipid[86]. The pH sensitive leakage from the liposome was only observed when (dioleoyl)phosphatidylethanolamine (DOPE) was used and inhibited when (dioleoyl)phosphatidylcholine (DOPC) was used instead[87]. The different headgroup positions the acylchains in DOPE in a more conical shape, while DOPC has a more cylindrical shape[88]. The unsaturated acylchains give DOPE a more exaggerated inverse conical shape (P>>1). DOPC has a P value closer to 1 and does not experience so much curvature stress that the membrane starts to leak. When the stabilizing lipid is protonated, the charge disappears and the headgroup gets less polar, which leads to a change in lipid shape and membrane destabilization. *See Figure 5.*

The best effect of this system was observed at pH 4.8[86,87] which is a hundredfold (2 pH units) lower than the tumor microenvironment. Although promising, it was already recognized at that time, that this mechanism is not sensitive enough to release significant amounts of drug in the tumor interstitium[86,87,89]. This led to a shift of focus to gene delivery, because the pH of the endosomes is much lower (5.0-6.0)[81,89–91]. Endosomes are transient organelles that sort and, if necessary, destroy internalized particles with degrading enzymes and acidification. Carriers that deliver genes to the cytosol (and/or subsequently the nucleus) must escape the endosome to avoid destruction. The release-trigger at pH ~5 and the fusogenic nature of DOPE makes these carriers perfectly suitable for delivery of siRNA or DNA plasmids. Now often used with CHEMS as pH-sensitive lipid[61,81,85,89,90]. Meanwhile, other release systems have been developed that are applicable in extracellular sites where the pH decrease is only one pH unit or less. Sheddable PEG-coatings are used, that are cleaved off at lower pH, to unmask targeting ligands or reactive surfaces of the liposomes. The PEG-coating allows the liposomes to avoid the RES and reach the tumor site but if they are unseen by the RES-cells, they cannot be seen by their target cells either. When PEG is attached to the liposomes with a hydrolysable linker, it can be cleaved off to expose targeting ligands or a positive surface charge (that interacts with the negatively charged cell surface of the target cells, but is toxic in the bloodstream)[90,92]. Especially the PEG2000-diortho ester–distearoyl glycerol (POD) linker is very suitable for hydrolysis in only slightly acidic environments[93,94]. The described particles consisting of POD:DOPE (1:9) had a similar stability as normal PEGylated liposomes in neutral pH but destabilized completely in environments with pH 5.0-6.0[93]. The PEG-shield not only protects the surface of the liposome but also increases stability, so when it is shed off, the liposome destabilizes more easily.

However, when looking for a smart trigger that is strong enough to quickly and completely release the drug load from a liposomal carrier, tumor pH appears not suitable. Apart from the fact that the decrease in pH is not big enough to provoke a conformational change in the stabilizing lipids, tumor tissue is often too heterogeneous. Not only is there a wide range of pH values between different tumors[84,85], there are big differences within different tumor areas as well[82,83,95]. Most problematically is that the most acidic areas were found to be furthest away from the supplying blood vessels and are out of reach for the circulating liposomes. Even if a more sensitive delivery system could be developed, the heterogeneity of the tumor would still cause unequal exposure to the drug. If a tumor associated trigger is used, it should be one that is more equally spread over all tumor areas.

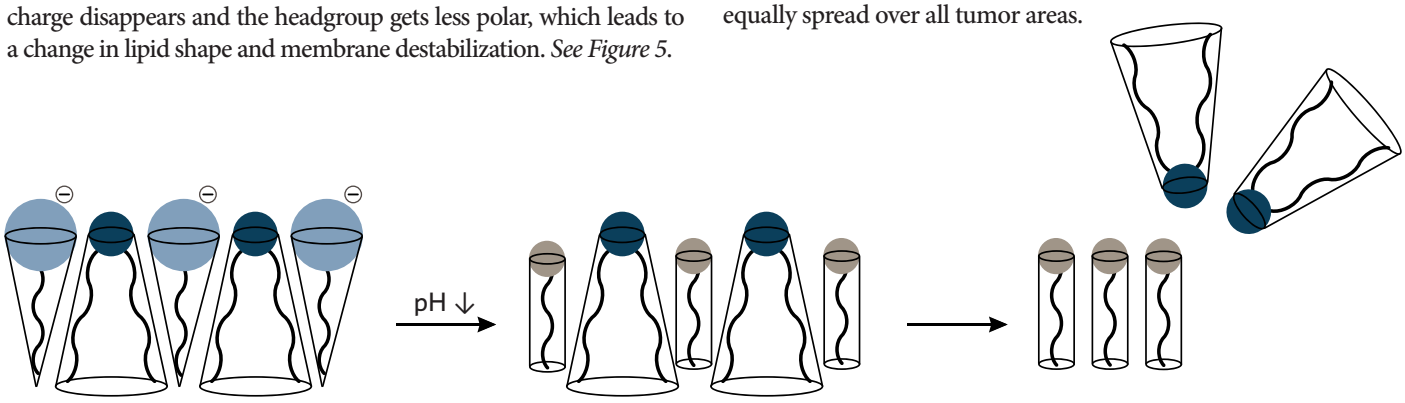

Figure 5 | Protonation of CHEMS destabilizes the membrane
When the charged headgroup of CHEMS is protonated, the relative hydrophobicity of the lipid is increased, which changes lipid shape. This increases membrane curvature stress and destabilizes the bilayer because the inverse conical shaped lipid adopts a non-bilayer conformation.

Phopholipase A_2 (PLA_2) mediated digestion of liposomes.

Another tumor environment-specific characteristic is the overexpression of phospolipases, like phospholipase A_2 (PLA_2)[96–99]. This enzyme catalyzes the hydrolysis of the ester bond of the *sn-2* acyl chain of phospholipids, producing a free fatty acid and a 1-acyl-lysophospholipid[100–102]. It is associated with pro-inflammatory states and overexpression of the enzyme may result in cell destruction or even organ failure[101,103,104]. LiPlasomes (from LiPlasome Pharma ApS, a Danish biotech start-up company) are an example of how smartly designed lipids are used to respond to the PLA_2 enzyme that is relatively specific to the tumor area[105–109].

As described in an earlier section, membrane permeability can be enhanced by destabilizing the membrane, by increasing membrane curvature stress. The addition of non-bilayer forming lipids to formed bilayers obviously increases curvature stress. Fatty acids (FA) and lysolipids (LL), are known to increase bilayer curvature stress and permeabilize the membrane[75]. When FA or LL were added to a stable membrane, the permeability of the bilayer increased, making it easier for drugs to pass, or leak through it. In higher concentrations the membrane completely dissolved[110]. When lipids in a membrane are degraded by PLA_2 they change *in situ* from phospholipids (cylindrical shape) to a lysolipid and a fatty acid (conical shapes), eventually dissolving the membrane. *See Figure 6.*

All phosphatidylcholines are sensitive to PLA_2, but the enzyme has to find access to lipid heads and it was found to be most active in membrane sections that have small defects or high curvatures[111–114]. The microstructure and PLA_2 activity on a supported membrane can be followed in real time using Atomic Force Microscopy[115,116]. Lipid layers sometimes form spontaneous curvatures that literally look like ripples in the membrane. It is unknown how they are formed but some researchers think it is a way to relieve packing frustrations in the membrane[116]. The ripple-phase can (temporarily) coexist with the other phases and is most prominent near the phase transition where there is already coexistence of the gel phase and the liquid-disordered phase[114]. This means that closer to the Tm, the system is more susceptible to PLA_2[108,114,116]. Changing lipid compositions makes membranes smoother and less susceptible to PLA_2 degradation[117]. SMPC is 1-stearoyl-2-myristoyl-*sn*-glycero-3-phosphatidylcholine that has two acylchains of different lengths and acts as an intermediate in lipid mixtures of two lipids with different lengths. Adding SMPC to distearoyl-phosphatidylcholine (DSPC) and dimyristoyl-phosphatidylcholine (DMPC) mixtures significantly increased the Tm and prolonged the lag-time of the system (the time from adding the enzyme to a burst release in experimental setting)[117]. Cholesterol has the same effect, through the same principle of relieving membrane stress and ordering the lipid tails as described before. When more than 20 mol% of cholesterol is added to the liposome, it becomes non-degradable by PLA_2[107,108]. This is the reason why Doxil® liposomes are not susceptible to PLA_2 degradation. For PLA_2 triggered release, adding cholesterol has an undesirable effect, but it also has the advantage that is better contains the loaded drug. In order to better retain drugs without introducing cholesterol, it can sometimes be desirable for some drugs to partition in the bilayer. When adding a negatively charged lipid, it can complex weakly basic (amine containing) drugs that will associate more with the membrane. When DSPC was substituted for distearoyl-phosphatidylglycerol (DSPG) that has a negative charge, it significantly improved the retention of the relatively hydrophobic drugs vincristine and ciprofloxacin in one study[118] and irinotecan and floxuridine in another[119]. This can be a method to achieve higher drug retention in the liposome, without having the PLA_2 resisting effect of cholesterol.

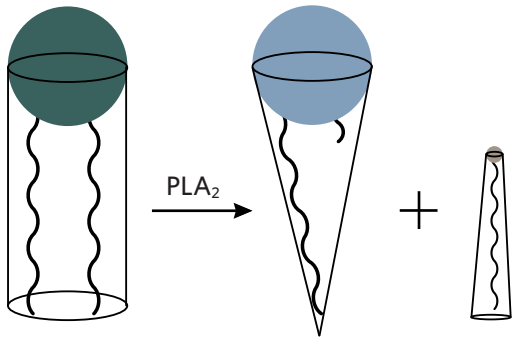

Figure 6 | Digestion of phosphocholines by PLA_2
When cylindrical shaped phosphocholines are digested by PLA_2, a conical shaped lysolipid and a separated acyl-chain are produced. Normally this is a free fatty acid, but it can also be a cytotoxic lipid of prodrug.

Interestingly, PEGylation has the opposite effect. Although it is assumed that PEG shields the surface from interaction with proteins or the RES cells, it actually increases the hydrolysis rate of liposomes by PLA_2[120,121]. The interaction of PE-PEG with the enzyme was reduced when the phosphate groups were methylated which shields the negative charge[121]. This indicates that PEG electrostatically attracts PLA_2 and high polymer densities and longer polymer chains greatly increased the hydrolysis rate[120].

This knowledge has been used to create liposomes that are optimally sensitive to PLA_2 and trigger unloading of the drug at the tumor site. LiPlaCis® is LiPlasome's formulation of cisplatin that is in the furthest stage of clinical development (although it was recently advised against continuation of Phase I studies because of a bad safety profile)[122]. It consists of DSPE-PEG-2000, DSPG and the main component DPPC. DPPC was chosen because it has a Tm of 42°C[74] which is close to body temperature and thus will have more lipid packing defects ("ripple phase") that promote PLA_2 activity.

The digestion of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) by PLA_2 yields one molecule of 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine (lysoPPC) and one molecule of palmitic acid (PA). This has a double effect. First, it destabilizes the membrane of the liposome, because the conversion of one cylindrical shaped lipid into two conical shaped lipids in the membrane introduces curvature stress and enhances membrane permeability. Both lipids have permeability enhancing effects, but together they work synergistically in 1:1 ratios. This is very convenient, because the enzyme produces lysoPPC and PA in equimolar quantities[110]. Secondly, when the liposomal membrane dissolves, the drug content is released, but there is also a burst release of membrane permeabilizing lipids that permeabilize other membranes. This was shown experimentally with a target membrane consisting of 1,2-O-stearoyl-*sn*-glycero-3-phosphocholine(1,2-di-O-SPC) that is inert to PLA_2 because of the ether bonds instead of the ester bonds. Similar to the target cells being inert to their own degrading enzymes[107]. The target membrane and PLA_2-sensitive liposomes were incubated together with PLA_2 and the target membrane was permeabilized, solely by the release of permeabilizing lipids from the PLA_2-sensitive liposomes. Addition of the hydrolysis products without the enzyme to the membrane confirmed the significantly increased effect when lysoPPC and PA were added together[107]

The permeabilizing effect of the lipid products on the target cells increases the efficacy of this delivery system because it enhances the uptake of the cytotoxic drug, but it can be taken another step further by incorporating cytotoxic lipids in the membrane. Some ether-lipids have known cytotoxic properties, but their use in

liposomes is limited because of the severe hemolytic effect after iv. administration[123,124]. By making a prodrug of these ether-lipids that can only be metabolized by PLA_2, there is limited systemic exposure and the active drug is only delivered to the tumor. The acyl chain at the *sn2* position is cleaved off by PLA_2 so if it is replaced by another cytotoxic compound, a double anti-cancer prodrug can be made[123–126]. The prodrug has to participate in the hydrophobic membrane barrier and it should not be too bulky so the choice is limited, but it can even be an advantage for poorly soluble drugs to be used this way. This has been done with chlorambucil[127], prostaglandins[128] and retinoids[126,129,130]. One of these retinoid prodrugs is 1-O-stearyl-2-RAR-C6-*sn*-glycero-3-phosphoglycerol (C6-RAR)[126]. The 4'-octyl-4-biphenylcarboxylic acid (RAR) compound is a selective agonist of the nuclear Retinoic Acid Receptor β2[131,132]. It is linked to the *sn2* position with a 6 carbon atom spacer because it was not hydrolysable when directly linked to the glycerol backbone[129]. When hydrolyzed, the C6-RAR compound and lyso-O-SPG (1-O-stearoyl-2-hydroxy-*sn*-glycero-3-phoshoglycerol are produced. The solubility of the prodrug is much better than the C6-RAR alone and the prodrug has no cytotoxic effect in the absence of PLA_2[129].

The prodrug forms stable liposomes on its own but is mixed with other lipids to enhance the susceptibility of the system to PLA_2. They were mixed with DPPC because of its Tm (42°C) and with DPPE linked to different lengths of PEG[125,126]. This significantly enhanced the susceptibility to PLA_2 and the release of C6-RAR when compared to liposomes of the prodrug only, that was not fully hydrolyzed after 7 days[125]. Addition of 10% DPPE-PEG750 optimally attracted PLA_2 and the introduction of DPPC introduced more surface heterogeneity and membrane defects as compared to single component lipid bilayers. Furthermore, hydrolysis of DPPC produces permeability enhancing lysolipids and fatty acids. Although more recent publications question whether LL and FA really permeabilize target cell membranes, it certainly helps the solubilization of the carrier membrane[125,133].

These double-prodrug systems where the lipid bilayer consists of a cytotoxic ether-lipid and a coupled anti-cancer drug can be a solution for the poor retention of soluble drugs in the aqueous core. But they could even be combined and form a triple action system when they are loaded with a traditional cytostatic like cisplatin or doxorubicin. In any case, these LiPlasomes are highly versatile systems that are a perfect example of how new insights in lipid polymorphisms and membrane behavior are exploited to create a powerful triggered release system.

However, there is one obvious disadvantage and that is that release is completely dependent of PLA_2 expression. Expression was seen in a wide variety of tumor types[96–99] but again tumors are heterogeneous and a high variation in response rates is expected. This does not have to be a problem because cancer therapy has always been based on the type of tumor. But the advantage of having little to no toxicity in tissues where PLA_2 is not present, turns into a disadvantage when there are tumor areas with very low expression. This is inherent to the use of a trigger that is tumor-specific. In the following chapters several external triggers will be discussed, that form a more steady and reliable trigger.

Photopolymerization, UV, visible light and NIR wavelengths

Low wavelength UV-light can be used as an outside trigger to disrupt the liposomal membrane and release its contents. Phospholipids can be engineered so that they polymerize after UV-radiation, which forms aggregates in the membrane bilayer and causes leakage. Paradoxically, polymerized liposomes have been investigated since the 1980s to increase membrane stability[134–138].

One of the earliest studied photopolymerizable lipids have a diacetylenemoiety in their acyl chains[135,137,139,140]. This makes them poorly water soluble and the ultrasonication needed for dispersion results in vesicle formation. These vesicles can be retained in the original formation by polymerizing their chains under UV-radiation of 254 nm when the chains are correctly aligned and hardly moving, so this is mainly limited to conditions below the Tm[135–138]. This contains the lipids in the gel-phase and inhibits transition to the liquid-phase (phase transition hysteresis), which significantly increases membrane stability and decreases leakage[137,138,141,142]. The polymerization results in poly(diacetylene) conjugated enyne bonds that absorb light in the visible spectrum which makes the solution undergo a transition from colourless via dark blue to red depending on the rate of polymerization[137,138,141].

Monomeric vesicles are extremely stable, but also mixed vesicles show an increased stability while remaining more flexible[143,144]. It was first thought that incorporating covalently cross-linked lipids in the membrane helped to increase the overall stability of the system. However, it turned out that when lipid mixtures were used, this led to phase separation, i.e. the formation of planes of extremely rigid polymerized lipids and more fluid planes of unpolymerizable (and/or unpolymerized) lipids[144–146]. This can lead to membrane leakage/destabilization in two ways. When two lamellar bilayer forming lipids are used (e.g. phosphatidylcholines), this does not have to compromise the stability of the vesicle because the bilayer will stay intact (however, if a lipid with a low Tm is used, the membrane could still become leaky). But when the non-polymerizable co-lipid prefers a non-lamellar phase, phase separation results in destabilization of the membrane and release of the aqueous content. DOPE was again very useful to create a triggered release system[147–149]. *See Figure 7.* Over time, many other polymerizable groups were incorporated in lipid chains, like acryloyl, methacryloyl, dienoyl, sorbyl, lipoyl, styryl and vinyl. It was also found that incorporating a polymerizable group in both acylchains results in crosslinking polymerization rather than linear polymerization, which gives a higher overall polymerization rate[150–154]. However, a lot of other biomolecules also absorb UV-light which makes it a less suitable trigger. Besides, UV-light can also have a destructive effect on healthy tissue, especially when higher intensities are used to penetrate deeper into the tissue. Therefore, photosensitive dyes have been incorporated in the bilayer, to catalyze the polymerization reaction in the visible spectrum. In more recent publications bis-SorbPC is widely used as polymerizable lipid, a phosphatidylcholine with a terminal hexa-2,3-dienoyl or sorbyl group in both acylchains that forms a crosslinked network[148,149,153,155,156]. This was combined with a group of lipophilic indocarbocyanine dyes that can be incorporated in the lipid bilayer. These dyes have absorption spectra in the blue, green and red regions of visible light and can all sensitize the polymerization process in presence of oxygen. This indicates that the polymerization reaction is initiated by the formation of hydroxyl radicals after excitation of the dye, in close proximity of the lipid tails[156]. The most effective sensitizer DiIC(18)3 or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine was also shown to be compatible with various bis-SorbPC containing long circulating PEG-liposomes, that also contained cholesterol to prevent "dark leakage"[157–159]. Longer wavelength light can also be used for photo-oxidizing synthetic lipids when a photosensitizer is incorporated. Photo-oxidation of ether-linked plasmalogen is similar to the acyl-chain cleavage by PLA_2 and also relies on enhanced membrane permeability or the induction of a non-lamellar phase. The oxidation can be triggered by sensitizers with absorbing

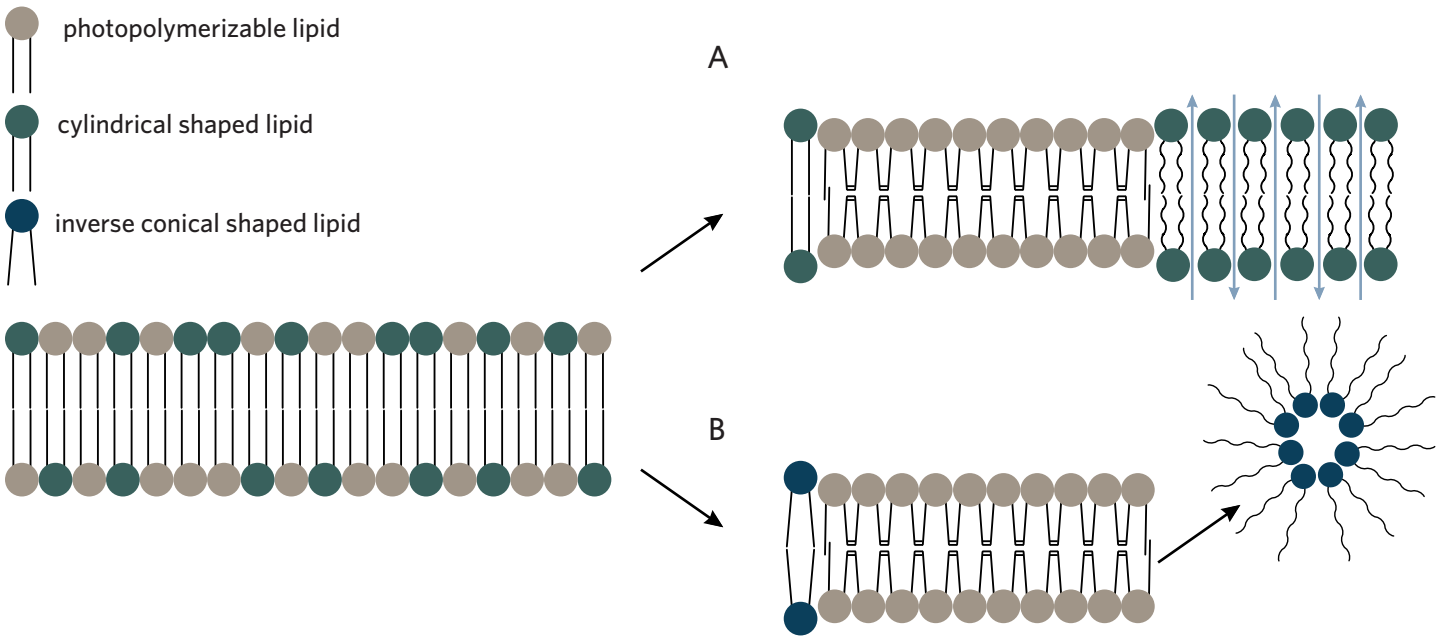

Figure 7 | **Destabilization by photopolymerization**
A. Two bilayer forming lipids. Phase separation leads to formation of two different lipid planes, if the co-lipid has a low Tm, the unpolymerized lipid plane can be fluid and leaky.
B. Co-lipid prefers non-bilayer phase. Phase separation leads to destabilization of the membrane, because the co-lipids adopt the inverse hexagonal phase.

wavelengths between 630 and 800, including zinc phthalocyanine, tin octabutoxyphthalocyanine and bacteriochlorophyll a[160,161]. Bacteriochlorophyll a sensitization produced the fastest release at 800 nm irradiation. These near-infrared (NIR) higher wavelength radiation can penetrate most tissues to depths of ≥0.8 cm, much deeper than short wavelength UV radiation[161–163]. The insufficient tissue penetration depth is probably the reason that none of the above described approaches has ever made it to in vivo experiments.

The advances in laser technology that allow a better control in wavelength, intensity and beam diameter have shifted the focus of medical and molecular imaging to higher wavelengths[164]. Recent publications have gone back to the earliest investigated polymerizablediacetylene groups, this time using a naturally occurring phospholipid $DC_{8,9}PC$ (1,2 bis(tricosa-10,12-diynoyl)-*sn*-glycero-3-phosphocholine)that forms tube-like structures in single compound solutions[139,140,165] and therefore had to be mixed with other lipids to form liposomes[166–168]. Attempts to photopolymerize $DC_{8,9}PC$ with a 514nm laser successfully released the entrapped calcein (Ex/Em, 485/517 nm), but not calcein blue (Ex/Em 360/460 nm) under identical conditions[168]. This led to the belief that also compounds entrapped in the aqueous core could act as a photosensitizer and that they not necessarily had to be incorporated in the lipid bilayer as described before with the indocarbocyanine dyes[157–159] and the Bacteriochlorophyll a[160,161]. Release from liposomes loaded with doxorubicin, that has an absorption spectrum matching the wavelength of the laser (DOX Ex/Em 490/590 nm), could also be triggered at tissue penetrating wavelengths[168]. Furthermore, it was already shown before that doxorubicin inside the target cells was more cytotoxic when it was excited by the 514nm laser[169,170].This has led to a whole different approach of using photosensitizable compoundsor nanoparticles to do damage to the cells, which has been reviewed recently[171–173], but this is beyond the scope of this review.

Light-triggered polymerization is a suitable mechanism to locally release the drug cargo, but the application of an external trigger to the tumor site often means that surgery is still necessary because of the limited penetration depth of light. In the next chapter, another trigger will be described that is non-invasive and optimally targetable, also to more deep-seated tumors.

Thermosensitive liposomes and MRI-guided HIFU

Using heat as a trigger for controlled liposomal release was first described by Yatvin *et al.*[174–176] who were also the first to propose the use of the lower pH in the tumor environment as a trigger[86]. They proposed the use of lipids with a Tm that was slightly above body temperature, so that phase transition would occur in the mildly hyperthermic circumstances in a heated tumor. Later it was shown that mild hyperthermia also increased the vascular permeability[177] and allowed deeper penetration of injected Evans blue dye[178] and monoclonal antibodies[179–182] into the tumor. The synergistic effect of hyperthermia and liposomes benefits from an increased permeability and the possibility to trigger release by mild heat[183–185]. The addition of MRI-guided high intensity focused ultrasound (HIFU) allows very accurate exposure of the tumor to mild heat to exploit the benefits of local hyperthermia in a very non-invasive manner[186–188].

The lab of David Needham at Duke University has optimized the formulation of doxorubicin loaded thermosensitive liposomes, to mild hyperthermic conditions (39-40°C) that are easily achieved in the clinic and reduced the release time to only tens of seconds[189,190]. The traditional long circulating thermosensitive liposome (TTSL) formulation was composed of DPPC, HSPC (hydrogenated soy phosphatidylcholine), cholesterol and DSPE-PEG-2000 in a molar ratio of 100:50:30:6 that had a 40-60% release at 42°C within 30 minutes[191,192]. Needham et al. made a lysolecithin-containing thermosensitive liposome (LTSL) that consisted of the lysolipid 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (MPPC), DPPC and DSPE-PEG-2000 in a molar ratio of 10:90:4 and released 50% of its doxorubicin content at 39-40°C in a matter of seconds[189,190]. The incorporation of the lysolipid in the bilayer lowers the Tm of the system because the membrane already starts to leak at temperatures slightly below the peak of the transition temperature because of pre-melting of the lipid interfaces[193,194]. Due to mismatches in lipid packing in the gel-to-liquid disordered transition phase, the membrane permeability is higher than in either the gel phase of the liquid disordered phase[193–195]. Another mechanism that increases the release of liposomes with this new composition is the fact that MPPC desorbs from the bilayer. DPPC and MPPC have matching headgroups and matching acylchains,

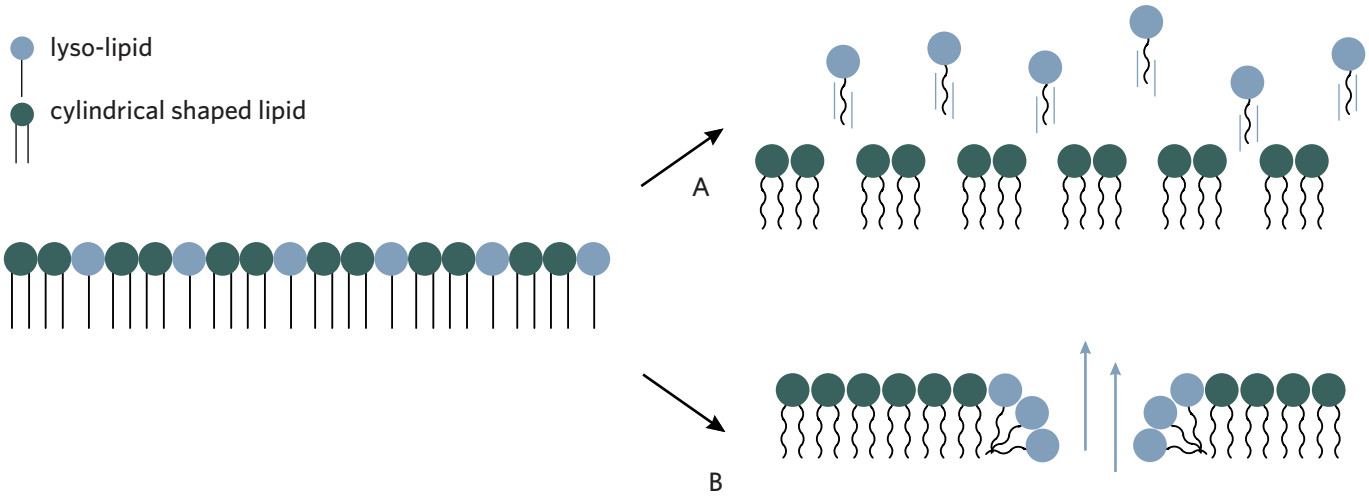

Figure 8 | Destabilization by chain melting
A. Lysolipids desorb from the membrane as soon as the first lipid starts to melt, making the membrane more permeable and leaky.
B. Lysolipids cluster together and adopt a micellar phase, creating pores in the bilayer.

but because the lysolipid has only one hydrophobic chain it is more water-soluble than DPPC. In the gel-phase the lipids are tightly packed and locked together by chain matching, but when the first lipids start to melt, MPPC prefers the aqueous phase and desorbs from the bilayer to eventually form micelles[189,196,197]. Another possibility is that the lysolipid adopts its preferred micellar formation at the phase boundaries of the bilayer that occur when the membrane starts to melt. This creates pores through which the contents can leak[198]. It is also very likely that both of these phenomena occur at the same time. *See Figure 8.*

This lowered phase transition temperature and the concomitant desorption of the lysolipid from the bilayer result in a rapid burst release in temperatures slightly above body temperature. The mild hyperthermia that is required for triggered release also increases the vascular permeability, greatly increasing the exposure of the tumor to the liposomal drug. In animal experiments a significant inhibition of a human xenograft tumor was shown when the doxorubicin loaded LTSL formulation was used in heated tumors[189,190,199]. The increased accumulation and the rapid and high burst release from the liposomes caused such high concentrations of doxorubicin inside the tumor that the blood flow was completely shut down[200]. In these animal experiments, the tumors were transplanted to the hind-leg that could be placed in a water bath for 1h. Of course, this smart release system calls for a more sophisticated trigger and moreover, application of heat via the skin is not possible for deep seated tumors.

The above described formulation of LTSL loaded with doxorubicin is being marketed as ThermoDox® by Celsion Corp (Columbia, MD, USA) for several indications in cancer treatment in combination with thermal ablation[198]. Image-guided thermal ablation was investigated as a possible alternative to anticancer surgery in small solid tumors[201]. This approach was based on the damage that is done by the heat induced coagulation necrosis inside the tumor, but perfusion cooling limits the tumor area that can reach the necessary 50-60°C[202,203]. This limits the affected area but also increases the chance of recurrence because cells in the outer perimeter are not killed. Therefore traditional chemotherapeutics were used as adjuvants to increase the coagulation diameter and tumor destruction. Non-thermosensitive liposomal doxorubicin (Doxil®) was already successfully used in combination with radiofrequency thermal ablation (RFA) (animal studies[204–207] and early human trial with hepatocellular carcinomas(HCC)[208]). After promising Phase I trials with ThermoDox®/RFA in unresectable HCC[209] Celsion could proceed directly to a pivotal Phase III 'HEAT' trial[210] that is expected to generate sufficient data to grant market admission in early 2013. In this combination doxorubicin greatly reduces recurrence rates as compared to RFA alone, because the outer margin of the tumor might not reach the sufficient temperatures to kill the cancer cells, but high enough to release the adjuvant doxorubicin.

There are also other thermal ablation methods such as microwaves, IR laser and high intensity focused ultrasound (HIFU). HIFU has recently gained a lot of attention because it is truly non-invasive and devices are becoming better and more widely available. HIFU uses ultrasound in higher frequencies and intensities than normal diagnostic ultrasound and is focused to small lesions (1-3mm) that reach temperatures high enough to cause immediate thermal toxicity[211]. However, when short pulses in short treatment cycles are used, only minor, non-lethal elevations (4-5°C) in tissue temperature are reached[212]. Pulsed HIFU was explored to improve the local delivery of various macromolecules including Doxil®[212,213]. In this case, the use of a thermo-sensitive formulation significantly increases therapeutic efficacy because damage is only done by the drug and release from the LTSL is much more complete than from Doxil® in this mild hyperthermic environment[214]. Imaging techniques have always been used as a guidance for thermal ablation devices[185], but recent advances in real-time 3D temperature mapping by magnetic resonance imaging (MRI) have opened up a great number of new possible applications[188,215]. This allows the safe use of thermal ablation in much deeper seated tissues, with the possibility to ablate bigger lesions while having less off-target exposure. Philips medical showed the clinical proof-of-concept for using MR-HIFU with thermosensitive liposomes that contained both doxorubicin and a fluorescent imaging marker[216]. The distribution of the intact liposomes could be followed in real time and an increase in fluorescence was seen in areas were release of the marker and co-encapsulated drug could accurately be triggered[187,216,217].

ThermoDox® is currently being investigated for additional indications in combination with MR-HIFU. This new technique allows truly non-invasive tissue heating, with an accuracy that was never possible before. This enables sophisticated planning of the therapy, where tumor tissue can be selectively heated to 42°C to enhance tissue distribution. The HIFU-mediated hyperthermia

can be temperature mapped where after the marker/cytostatic liposome can be administered and tumor accumulation can also be followed in real-time, or tracked after a couple of days. The tumor area can then be heated again to trigger release and drug delivery can once again be imaged by MR[186].

The thermosensitive liposome formulation is another good example of how the lessons in lipid polymorphisms can be used to design smart lipid systems with targeted release. In combinations with the advances that medical technology provides with the non-invasive imaging and heating-system a real step forward can be made. The versatile MR-HIFU system might be the "smart" trigger that can really take triggered release to the next level.

Final remarks and experimental approaches

The previous chapters described the most commonly used mechanisms that are used for triggered release. It was shown that there are two factors of major importance: first, a trigger that is highly specific for the tumor and equally spread over the tumor. And second, a mechanism to quickly destabilize the membrane at the target site, while limiting leakage in the circulation.

A more experimental approach to also deliver the trigger to the tumor is described by Cheong *et al.* where they colonize the tumor with bacteria that express a special enzyme[218,219]. It was already known that anaerobic bacteria could thrive in the hypoxic and immune-privileged core of a tumor, the *Clostridium novyi* strain in particular[220]. Administration of wild-type *C. novyi* however was very toxic to test animals, but was very well tolerated when the α-toxin gene was deleted. Colonization of a tumor with *C. novyi-NT* (non-toxic) alone resulted in severe necrosis but was not enough to fully eradicate the tumor. Interestingly, *C. novyi-NT* was found to express a novel lipase that was dubbed Liposomase because of its capability of digesting liposomal membranes[218,221]. This principle was shown in combination with Doxil® and fully cured 70% of the test animals[218,219]. The number of administered bacteria could theoretically be lowered to an amount where only one CFU ends up in the tumor, because it will multiply and produce the enzymatic trigger at the target site. However, it is not very likely that infection of patients with a genetically modified bacteria prior to treatment will be clinically approved, which makes this a less feasible, but nevertheless very interesting approach.

The second important factor is the ability to quickly and completely destabilize the membrane to release the drug cargo. The problem with many triggered release systems is that they have to have an inherent instability that causes leakage in the circulation. For example, in attempts to create more stable liposome systems, lipids with a high Tm were used to increase stability at 37°C. However, both the thermosensitive formulation and the LiPlasomes benefit from the lipid disorder that occurs around the phase transition that is not slightly above body temperature. In almost all cases, the adaptations needed to create a triggered release system compromise the systemic stability, causing preliminary leakage.

A different, experimental approach uses bacteria-derived channel proteins in the liposomal membrane that can be opened and closed by an external trigger[222–225]. Koçer *et al.* engineered the mechanosensitive channel of large conductance (MscL) from Escherichia coli to respond to UV-light[222] or changes in ambient pH[223]. The MscL normally only responds to extreme membrane tension and opens as a large nonselective pore of 3 nm through which ions, solutes and small proteins can diffuse. By coupling a reactive group to the hydrophobic pore region of the subunits, a charge build-up can be induced by a change in pH or by UV-radiation. This leads to a conformational change that opens the channel protein allowing the content to leak out of it and the channel can be closed again by illumination with visible light, or by changing the pH again[222–225]. This is an interesting approach because it uses a valve-mechanism that is independent of membrane structure and composition. It could be combined with an impermeable membrane because the release is not dependent on membrane destabilization but on the opening of the nanovalve. In vivo applications are not published yet, but because they are derived from bacteria these proteoliposomes might be very immunogenic. Still, this is a very interesting approach that overcomes the problems that are associated with membrane destabilizing mechanisms used for triggering release.

Conclusion

Sterically stabilized long-circulating liposomes are capable of extravasating through the leaky tumor vasculature and accumulate in the tumor interstitium. Recent progress in lipid and membrane studies have led to such stable liposomes that leakage in the circulation is minimal, but release at the target site is also far from complete. Therefore, mechanisms to destabilize the membrane at the target site were explored, in order to create trigger-specific release systems. In most cases, the membrane destabilizing mechanism compromises the stability of the carrier in the circulation, so it will always stay a trade-off between systemic stability and triggered release.

In this review different release mechanisms and triggers are described. When it comes to smart lipid design, the PLA_2-sensitive liposomes (LiPlasomes) are definitely the most sophisticated system. The lipid composition is changed in such a way that full digestion of the membrane by PLA_2 only takes minutes. Furthermore, the lipid-prodrug construction makes systemic toxicity almost impossible. Because the liposomes are empty and the drug is only metabolized at the tumor site, no harm can be done, even if lipids would preliminarily desorb from the membrane in the circulation. As mentioned before, the drawback of this mechanism is that metabolization to the active drug is limited by PLA_2 expression.

An external trigger is more reliable and can be exclusively applied to the tumor site. Radiation, light and heat are successfully used as trigger systems, but these often still need (minor) surgery to apply. In this aspect, the MR-HIFU system is by far the smartest and most advanced trigger. It combines high resolution imaging with a non-invasive trigger that can selectively heat the tumor with very high accuracy. This versatile system not only permits treatment of deep-seated tumors with thermo-sensitive liposomes, it also improves treatment by enabling careful planning based on real-time imaging. The pulsed HIFU can first improve the permeability of the tumor vasculature and then trigger a heat-mediated release, after which tumor distribution can be monitored in real-time.

The combination of such smart imaging and trigger systems with smart liposome design can further improve the delivery of small-molecule drugs to the tumor environment, while limiting toxicity to other organs and healthy tissue. ■

Strategies for triggered drug release from liposomes: In search of "smart" lipids and "smart" triggers.
Master thesis (7,5 ECTS) for Master Drug Innovation
by Erik Oude Blenke.
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October 2012

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